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Detection by Replica Plating of False Revertant Colonies Induced in the Salmonella-Mammalian Microsome Assay by Hexavalent Chromium

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The replica plating method as developed by Lederberg has been used to differentiate between "true" and "false" histidine-requiring revertant bacterial colonies which develop on minimal agar plates in the Ames test. Strains of *S. typhimurium* LT2, TA 100, when exposed to either sodium dichromate or the fumes from the welding of stainless steel, develop colonies whose apparent numbers are directly in proportion to the Cr(VI) content per plate in both cases, over a wide dose range. Replica impressions of the resulting colonies were transferred to Vogel Bonner minimal agar plates and incubated for 48 hr at 37°C. It was then observed that considerable numbers of "false" revertant colonies were obtained at those Cr(VI) doses which resulted in a pronounced toxic effect, albeit with an acceptable level of the bacterial background lawn. No morphological distinction between "true" and "false" revertant colonies could be made. Although it would appear that at low doses (i.e., low toxicity) the true mutagenicity of stainless steel welding fumes can be completely accounted for by the presence of Cr(VI), the dose range over which the mutagenicity assay is reliable cannot be estimated from examination of the background lawn or from an estimate of the degree of survival of the treated cultures. Thus there is raised a serious question concerning the reliability of quantitative data published in bacterial mutagenicity testing where replica testing of the histidine requirement of the resulting "revertant" colonies is not routinely made. It is suggested that the replica technique can easily be developed as a simple and useful tool for the control of histidine requirement and ampicillin resistance in routine mutagenicity testing. Difficulties in transferring separate colonies from densely packed plates may, however, place a limit on the practical application of the method when used in quantitative analyses.

Introduction

The Ames test is widely used to assess the mutagenic potential of chemicals (*1*). The principle of this assay is based on the properties of inducing reverse mutations in histidine requiring auxotrophe *Salmonella typhimurium* LT2 strains. The reverted bacteria are detected as colonies on minimal agar plates. Although current attempts at standardization of this assay do not include a procedure for controlling prototrophy of colonies developed on minimal agar plates, the use of a replica technique to confirm the lack of histidine requirement has been previously recommended (*2*).

Dichromates, chromates and chromium trioxide have all been found mutagenic in different bacterial mutagenicity assays (*3*, *4*), and in the Ames test they induce base-pair substitutions as well as frameshift mutations (*5*). It is also well documented that fume particles from metal arc welding on stainless steel have mutagenic activity (*6*, *7*) primarily due to their Cr(VI) content (*8*). In this study, the replica technique, as developed by Lederberg (*9*), is used to differentiate between "true" and "false" revertant colonies on minimal agar plates in the Ames test, for a series of test materials containing Cr(VI), in order to determine if the replica technique might be a useful tool in verifying histidine requirement when substances with a pronounced toxic effect are tested.

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Materials and Methods

Test Materials

The welding fumes—$Na_2Cr_2O_7$, MMA/SS (Manual Metal Arc/Stainless Steel) welding fume and MIG/SS (Metal Inert Gas/Stainless Steel) welding fume—from a series described by Stern and Pigott (*10*), were produced and collected as described by Stern (*11*) and subsequently dissolved in sterile demineralized water and exposed to ultrasound for 5 mins. The Cr(VI) content is assumed identical to the water-soluble Cr content measured by AAS.

Mutagenesis Assay

The Salmonella/mammalian microsome mutagenicity test (plate incorporation assay), using the tester strain TA100 of *Salmonella typhimurium*, was performed as described by Ames et al. (*12*) with the following modification: 0.4 mL of an overnight culture of TA 100 was transferred to 20 mL nutrient broth and incubated 6 hr at 37°C before use. The addition of S-9 mix was not included in the test series. Upper dose levels were chosen as 50 μg of $Na_2Cr_2O_7$ and 50 μg water-soluble chromium in the welding fumes.

Replica Technique

The replica technique as developed by Lederberg (*9*) was used to transfer copies of one plate of each dose to Vogel-Bonner Medium E plates. Sterilized velvet with a dense nap and mounted on a wooden block was used as contact material.

Results

The number of revertant colonies per plate (average of three plates) induced by suspensions of $Na_2Cr_2O_7$ and of MMA/SS and MIG/SS welding fume particles, as a function of weight of $Na_2Cr_2O_7$ and of water-soluble chromium, per plate is shown in Figures 1-3. The number of colonies on one of the master plates and on each of the respective replicas as a function of dose is listed in Table 1 and plotted in Figures 1-3 as well.

The background of bacterial growth was clearly observed to be inhibited at 50μg/plate of all sub-

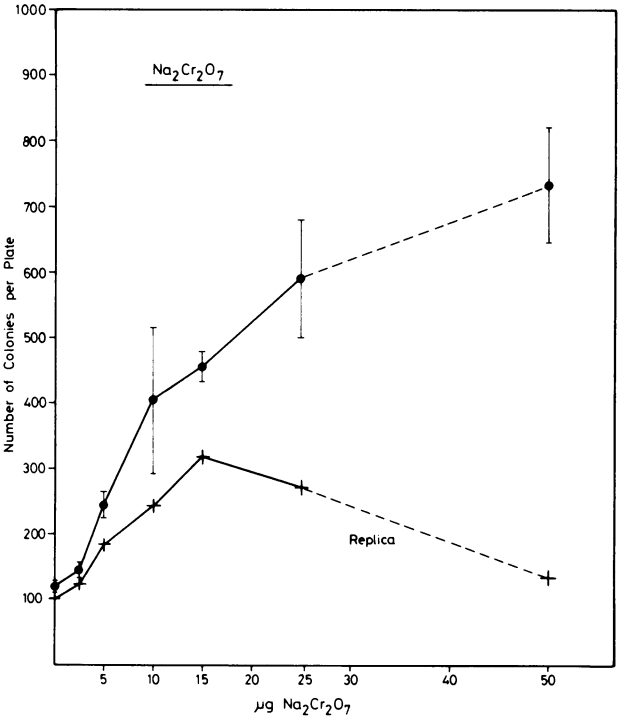

FIGURE 1. The average number of colonies per plate (from three plates ± 1 SD) vs. the concentration of $Na_2Cr_2O_7$. The number of colonies on replica plates transferred from one of the master plates is shown (see Table 1). Note that although the initial slope of the dose revertant curves indicates a response to Cr(VI) from dichromate which is three times that from MMA/SS fumes (Fig. 2), the corresponding slopes of "true" revertants vs. Cr(VI) from replica plating are almost identical for dichromate, MIG/SS (Fig. 3) and MMA/SS (Fig. 2).

Table 1. Induction of mutations in *Salmonella typhimurium* TA 100 by $Na_2Cr_2O_7$, MMA/SS and MIG/SS welding fumes and application of the replica technique in controlling the phototrophy of colonies developed on Vogel-Bonner Medium E.

Cr, μg per plate[a]	Number of colonies per plate					
	$Na_2Cr_2O_7$		MMA/SS		MIG/SS	
	Master plate	Replica plate	Master plate	Replica plate	Master plate	Replica plate
0+[b]	110	103	110	103	110	103
2.5	139	125	175	125	217	177
5.0	272	185	215	146	332	215
10.0	304	244	372	265	648	376
15.0	442	320	534	372	472	275
25.0	544	273	692	337	688	240
50.0	688	135	–	–	888	194

[a] μg $Na_2Cr_2O_7$ per plate or μg water-soluble chromium in MMA/SS and MIG/SS per plate.
[b] Control.

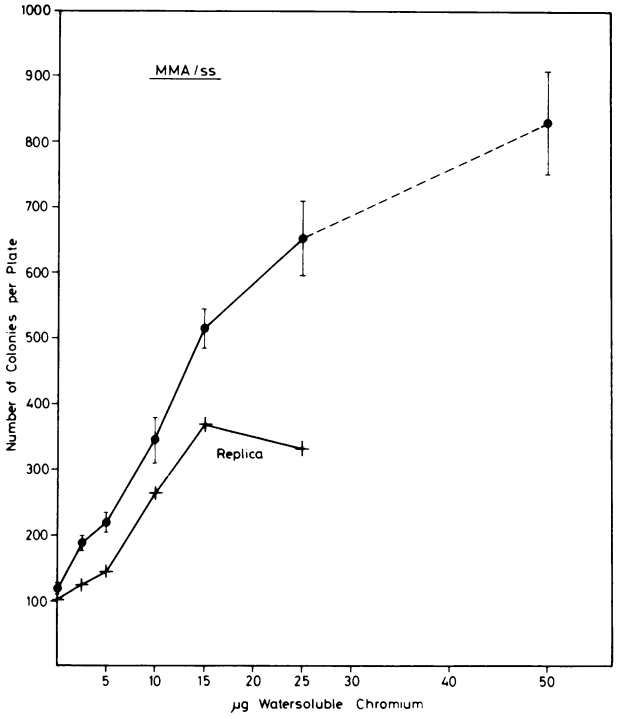

FIGURE 2. The average number of colonies per plate (from three plates ± SD) vs. μg water-soluble chromium in MMA/SS fume.

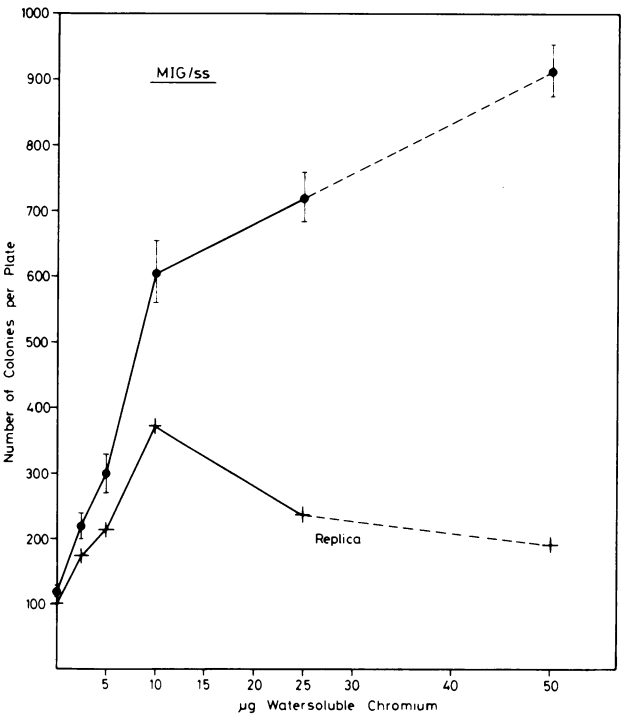

FIGURE 3. The average number of colonies per plate (from three plates ± SD) vs. the μg water-soluble chromium in MIG/SS fume.

stances tested. An apparent weak inhibition was found at 25 μg/plate.

All Cr(VI)-containing substances tested exhibit a clear dose-related induction of revertant colonies. On the average, 10 μg $Na_2Cr_2O_7$, 9 μg water-soluble chromium in MMA/SS fume and 5 μg (water-soluble) chromium in MIG/SS fume per plate account for a doubling in the number of "true" revertant colonies.

On the replica plates, 65-85% of the number of colonies on the master plates could be recognized as separate colonies at dose levels up to 15 μg/plate. A considerable drop in the number of replicated colonies was found at 25 and 50 μg/plate.

Discussion

Metals such as chromium form electrophilic ions in solution and are able to react with nucleophile materials such as the N-bases in DNA. This characteristic appears to be an essential feature of substances with mutagenic properties. It is well documented that materials containing Cr(VI) have mutagenic activity in many different short-term mutagenicity tests, and the present investigation is a further confirmation of the dose-related induction of mutations in bacterial assays (*13*). It is, however, often rather difficult to compare data from different investigations on complex materials such as welding fumes, because the results are only rarely expressed as a function of specific active substances (such as amount of water-soluble chromium per plate) and collecting techniques are often very different in different laboratories.

Dichromates and welding fume particles have a pronounced toxic effect on the tester strains of *Salmonella typhimurium*. An examination of the background lawn of bacterial growth is routinely used to determine the presence of toxic effects and the limit of toxicity. By using this practice in the present investigation the limit of toxicity would have been fixed at 25μg $Na_2Cr_2O_7$ or 25 μg water-soluble chromium per plate: Stern et al. (*8*) have chosen 20 μg/plate Cr(VI) as a standard upper dose level.

The colony count of the replica impressions shows a distinct drop at 25 μg/plate compared to 15 μg and corresponds to 30-50% of the number on the master plates. There are, therefore, a considerable number of "false revertant" colonies on the master plates at 25 μg/plate of all three test substances.

It is well known that massive killing of bacteria on the minimal agar plates permits the development of small colonies of histidine-requiring bacteria because of the large amount of histidine available per vital bacterium on the media (*12*).

On the master plates of this study it was not possible to make a morphological distinction between different (i.e., false and real) revertant colonies at 25 μg

Cr(VI); at this dose the background lawn was not inhibited to any appreciable extent.

The results of these preliminary investigations suggest that it might be necessary to recommend that prototrophy of the colonies developed on the minimal agar plates be controlled in standard procedures, especially when dealing with test materials with a distinct toxic effect on the tester strains. Examination of the background lawn of bacterial growth might not always be a reliable method of evaluating the influence of toxic effects on the test system, and an estimation of the number of surviving bacteria should at least be included in all routine analyses. The application of the replica technique has a limitation in the practical difficulties of transferring separate colonies from densely packed plates, and the results at high colony counts yield only qualitative information. With some experience in handling the technique it should be a simple and useful tool in the control of histidine requirement and ampicillin resistance in bacterial mutagenicity testing.

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